Chapter

Novel Hemoglobin from *Synechocystis* sp. PCC 6803: Shedding Light on the Structure-Function Relationship and Its Biotechnological Applications

Mohd. Asim Khan, Sheetal Uppal and Suman Kundu

Abstract

Cyanobacteria are oxygenic photosynthetic prokaryotes, practically present in every plausible environment on the earth. In 1996, the first cyanobacterial genome was sequenced from *Synechocystis* sp. PCC 6803 and the cyanobacterial genome database has been continuously growing with genomes from more than 300 cyanobacterial and other related species, so far. *Synechocystis* sp. PCC 6803 is one of the best-characterized cyanobacteria and has developed into a model cyanobacterium that scientists are using throughout the world. At the same time, the field of hemoglobin was undergoing a breakthrough with the identification of new globins in all three kingdoms of life including cyanobacteria. Since then, the newly identified globins in the cyanobacteria are raising intriguing questions about their structure and physiological functions, which are quite different from vertebrate's hemoglobin and myoglobin. These hemoglobins have displayed unprecedented stability, unique heme coordination, novel conformational changes, and other properties that are not often observed in the globin superfamily. This chapter provides an overview of the unique globin from *Synechocystis sp.* PCC 6803, its interacting protein partners, proposed functions, and its biotechnological implications including potential in the field of artificial oxygen carriers.

Keywords: Cyanobacteria, *Synechocystis* sp. PCC 6803 hemoglobin, Structural features, Heme stability, Physiological function, Biotechnological application

1. Introduction

The ancient cyanobacteria played a fundamental role in changing the composition of the early, oxygen-poor reducing atmosphere into an oxidizing atmosphere of the earth. These tiny oxygenic phototrophs inhabit varied ecosystems and habitats ranging from oceans to hot springs and deserts [1]. They can also be found in extreme environments, such as acidic bogs and volcanoes. The plethora of available

information on the diversity and physiology of cyanobacteria provides an excellent base for exploring their application in biotechnology. Because of their ability to harvest solar energy and convert atmospheric CO_2 to useful products like biofuels and bioactive compounds, they serve as a promising organism which is used for medical treatments and various industrial applications [2].

Oxygen provides an enormous source of energy for biological functions; however, it can also be toxic to organisms. It is believed that cyanobacteria were among the earliest prokaryotic organisms responsible for the oxygen-rich environment on the young planet earth and currently, nearly 99% of the oxygen is contributed by the eukaryotic algae [3]. It has been revealed that all the eukaryotic phototrophic organisms derived the ability to produce oxygen during photosynthesis through endosymbiosis [4]. Later, it was discovered that the heme-containing protein that sequesters and protects the primitive cyanobacteria cells from toxic O_2 is almost identical to the energy-generating apparatus in the photosynthetic bacteria [5]. The basic chemical apparatus became increasingly complex through time and evolution, however, the interaction between the metal atom in the porphyrin ring and the oxygen remains unchanged [6]. The heme-containing proteins form a large class of macromolecules that have diverse and distinct biological functions. Comparative analysis of hemoproteins revealed that the changes in the amino acid sequence and their interaction with the porphyrin ring mainly involved in the multitude of different functions which include electron-transferring cytochromes, intracellular peroxidases, and lignin-degrading extracellular peroxidases. The well-studied heme-binding proteins are vertebrate hemoglobin (Hb) and myoglobin (Mb). The heterotetrameric hemoglobin is present at high concentration (15 g/100 ml) in normal human blood and involved in the oxygen transport in the circulatory system whereas myoglobin is a monomeric oxygen storage protein mainly located in the cardiac and striated muscles [7].

Recent breakthroughs in molecular biology tools and genome sequencing techniques led to the identification of hemoglobin genes in almost all kingdoms of life including plants, animals, bacteria, and fungi. Extensive bioinformatics surveys of available genomes identified putative globins with several characteristics that are distinct from the classical globins [8]. These distinct globins have been designated as "novel" or "new" globins to distinguish them from the traditional globins. These new Hbs display differences in the coordination chemistry of heme Fe atom in which all six coordination sites are occupied in the absence of exogenous ligand and referred to as "hexacoordinated hemoglobins (HxHbs)" compared to "pentacoordinated" heme Fe coordination chemistry of classical vertebrate Hb and Mb [9, 10]. Another set of novel hemoglobins have been discovered which are 20–40 amino acid residues shorter than the mammalian hemoglobins, resulted in modification of the canonical "3-on-3" globin fold and provided them with a shortened "2-on-2" globin fold [11–13]. These classes of hemoglobins are called "truncated hemoglobins (TrHbs)" and constitute a major class of the globin family [14].

In 1992, the identification of truncated hemoglobin in the nitrogen (N_2)-fixing cyanobacterium *Nostoc commune (N. commune)* strain UTEX 584 opened the avenues for exploring and identification of hemoglobin gene in cyanobacteria and green algae [15]. Several years later, genome sequence analysis of the first photosynthetic non-N_2 fixing *Synechocystis* sp. PCC 6803 (S6803) revealed a single copy of the globin gene [16]. Among all cyanobacterial species, S6803 is one of the most widely studied cyanobacteria. In this article, we will provide an overview of the history of *Synechocystis* hemoglobin (*Syn*Hb), its proposed physiological functions and interacting partners as well as the biotechnological applications of the cyanobacterial hemoglobin in the designing of artificial oxygen carriers.

2. *Synechocystis* sp. PCC 6803 hemoglobin: an unusual hemoglobin from a cyanobacterium

S6803 was the third unicellular prokaryotic and first non-diazotrophic photosynthetic organism whose genome is completely sequenced. The genome sequence analysis showed the presence of a single hemoglobin gene (coded by slr2097 gene; named as glbN), encoding 123- amino acid polypeptide chain sharing 55% sequence identity with the cyanoglobin from *N. commune* [17]. Compared to *N. commune*, the location of the slr2097 gene in the genome does not provide any indication of a functional role for the protein. So, to uncover the physiological role, this cyanobacterial globin is being investigated worldwide and a series of research efforts by several pioneers provided the structural and functional understanding as outlined in **Figure 1**.

2.1 Biochemical and structural features of *Synechocystis* Hemoglobin

In the year 2000, two different groups reported the preliminary biochemical characterization by cloning and over-expressing the slr2097 globin gene in *E.coli* [17, 18, 32]. The molecular weight of *Syn*Hb protein is around ~13 kDa and was found to be a stable α-helical monomeric protein [33]. This cyanobacterial globin is "hexacoordinated" in which His46 (distal) and His70 (proximal) function as internal heme iron axial ligands [19]. Comparative sequence analysis of *Syn*Hb with pentacoordinated sperm whale Mb and other truncated hemoglobins (trHbs) showed that *Syn*Hb is a member of the truncated hemoglobin superfamily with "2-on-2" globin fold (**Figure 2**). The overall crystal structure of *Syn*Hb is almost similar to other trHbs and the NMR structure, however, some conformational changes were found, which was partly attributed to the presence of a unique third covalent linkage between the heme-2-vinyl and the Nε2 atom of His117 residue – a feature observed in crystal structure but not in the NMR structure. This unique post-translational modification with heme moiety is not detected in any other globins discovered so far. Interestingly, *Syn*Hb displays the characteristics features of both trHbs and HxHbs. It has been found that His46 residue covalently attached to the heme Fe atom on the distal side resulting in "hexacoordination". However, unlike in other HxHbs whose structures are solved, His46 occupies E10 position compared to common E7 position and not involved in the stabilization of the bound ligand (**Figure 3**). Interestingly, structural comparison of *Syn*Hb with other trHbs

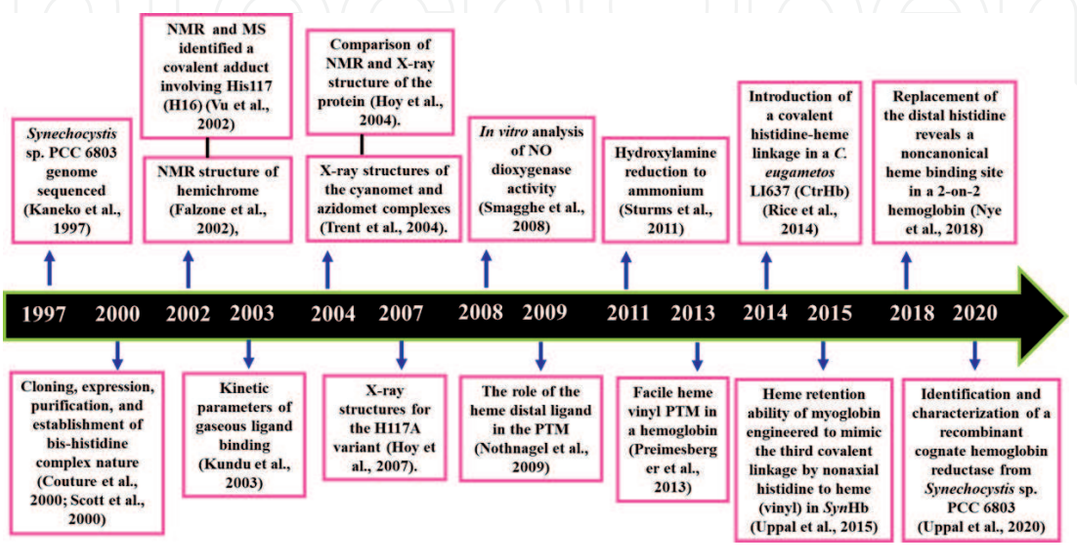

Figure 1.
Timeline of milestones in Synechocystis *hemoglobin research [16–31].*

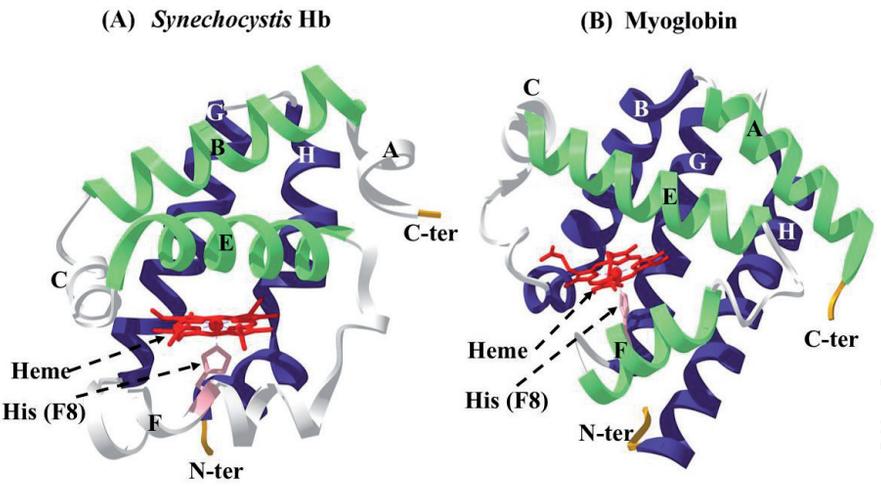

Figure 2.
Three-dimensional structure showing globin fold. A) a typical truncated Synechocystis *hemoglobin (PDB ID: 1RTX) showing "2-on-2" globin fold (BE (green)/GH (blue)). B) Three-dimensional structure of classical globin i.e. myoglobin (PDB ID: 5MBN) showing "3-on-3" globin fold (AEF (green)/BGH (blue)).*

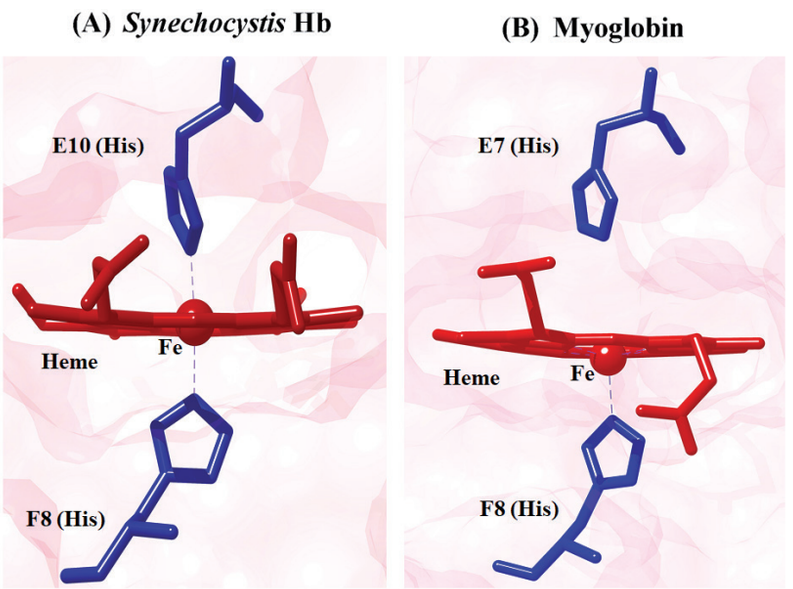

Figure 3.
A) Three dimensional structures of Syn*Hb (PDB-1RTX) shows hexacoordinate heme coordination chemistry with distal his (E10) and proximal his (F8) covalently linked to heme Fe atom. B) Three dimensional structures of Mb (PDB- 5MBN) shows pentacoordinate heme coordination chemistry with distal his (E7) not directly bound to heme iron atom whereas proximal his (F8) covalently linked to heme Fe atom.*

reveals the absence of a ligand tunnel, a characteristic feature of trHbs connecting the distal heme pocket to the solvent. The tunnel formation is observed post-ligand binding in *Syn*Hb and has been proposed to facilitate ligand escape from the heme pocket. *Syn*Hb provides the first crystal structure of a trHb in both unliganded and ligand-bound state [19, 34]. Among all the available hemoglobin structures, *Syn*Hb is the only globin which undergoes a significant conformational change in the tertiary structure upon ligand binding in hexacoordinated-*Syn*Hb [20].

2.2 Role of key residues in the stability and folding of *Synechocystis* Hemoglobin

Preliminary investigation of *Syn*Hb by Hoy et al. [35], and Nothnagel et al. [21], revealed that this cyanobacterial Hb is naturally a very stable Hb. Their work highlighted the role of the unusual third His (His117) in imparting extra heme stability

in *Syn*Hb. Though the globin has been reported to be stable, negligible reports exist as to its relative stability, extent of stability, the factors that contribute to it and their applications and thus *Synechocystis* hemoglobin is being investigated worldwide. In the past few years, Kundu's laboratory is intensively involved in studying this unique cyanobacterial globin to understand the biophysical traits that define this globin and structure–function relationship in comparison to classical Hbs. Extensive mutational studies and heme loss assay reiterated and validated some earlier propositions that the third covalent linkage between heme-2-vinyl group and His117 residue is the major holding force for heme in *Syn*Hb [22, 32]. Several key residues near the heme pocket have identified that influence the structural integrity of protein and thus play vital role in the cyanobacterial globin expression and synergy of amino acid side chains in the heme and polypeptide stability (**Figure 4**) [33]. Their studies have revealed several interesting findings which include: 1) His117 is indispensable for heme retention, while either of distal His46 or proximal His70 is required for heme uptake by apo-*Syn*Hb (globin protein without heme); 2) Acid-induced denaturation studies showed that *Syn*Hb did not release heme from its protein matrix and displayed features of a molten globule state at pH 2.0; 3) Acid- and chemical-induced denaturation studies revealed that none of the heme pocket residues affect the polypeptide stability except His117 residue; 4) This cyanobacterial Hb is extremely thermostable compared to classical pentacoordinated Hbs and thermal unfolding is affected both by distal His46 and His117 residues. Based on these findings, it has been proposed that *Synechocystis* Hb could be used as a model system for understanding the protein folding and stability of a new class of hemoglobins.

2.3 Ligand binding kinetics in *Synechocystis* Hb

The reaction of *Syn*Hb with O_2 and CO is quite unusual. The association rate constants for O_2 and CO is partly similar to the hemoprotein (glbN) from the *N. commune* cyanobacterium [36]. Compared to other hemoglobins (**Table 1**), such as Mb and human Hb, soybean leghemoglobin (Lba), and non-symbiotic hemoglobin (nsHb) from rice, these two cyanobacterial proteins have remarkably large values for most rate constants [38–42]. The two cyanobacterial Hbs are different in their O_2

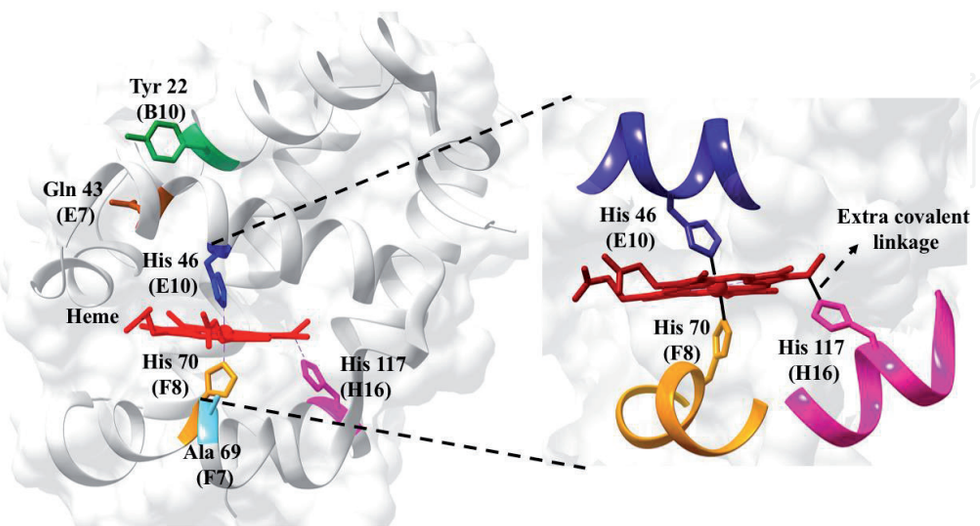

Figure 4.
Structural representation of Synechocystis *hemoglobin (PDB ID: 1RTX). The truncated globin fold (2-on-2) is represented in gray and the heme moiety in red. Several key residues in the heme pocket are displayed in color. The three histidines covalently associated with heme are shown in zoomed section. His46 and His70 directly coordinate to heme iron and constitute "hexacoordination". The third His (His117) is covalently associated to heme vinyl group.*

Hb	k'_{O2} ($\mu M^{-1} s^{-1}$)	k_{O2} (s^{-1})	k'_{CO2} ($\mu M^{-1} s^{-1}$)	$k_{entry, CO}$ ($\mu M^{-1} s^{-1}$)
Synechocystis hyobin	240	0.014	90	
Nostoc commune (glbN)	390	79	41	
M. tuberculosis Hb (HbN)	25	0.2	6.8	
Rice Hb (rHb1)	68	0.038	6	
Sperm whale Mb	17	15	0.5	17
Human Hb α- chain	23	11	2.9	11
Human Hb β- chain	79	28	7.1	11
Soybean LegHb (Lba)	130	5.6	17	320

k_{O2} = association rate constant (on-rate); k_{O2} = dissociation rate constant (off-rate); k'_{CO2} = association rate constant (on-rate); $k_{entry,CO}$ = rate constant for entry through the protein matrix.

Table 1.
Rate constants for ligand binding to several wild type hemoglobins [21, 37].

dissociation rate constants. The oxygen (O_2) dissociation rate constant of *Syn*Hb is very low compared to faster O_2 dissociation rate of glbN (**Table 1**). The low dissociation constant of *Syn*Hb is similar to other HxHbs like rice Hb1. All classical Hbs have a relatively higher off rate. *Syn*Hb exhibits large association rate constants for CO and O_2 which is because of unusual reactive heme iron, suggesting that the ligand is trapped in the heme pocket which increases the chance of bond formation rather than escape (**Table 1**). Thus, bimolecular rate constants cannot surpass the rate constant for ligand entry through the protein matrix. It was observed that k'_{CO} for *Syn*Hb is larger than $k_{entry,CO}$ for Mb and human Hb; thus indicating that the heme pocket of *Syn*Hb is highly solvent-exposed. The unusual ligand binding kinetics in *Syn*Hb may not support a role in O_2 transport and storage. In trHbs, the conventional "histidine-gate" path for ligand binding is blocked [43]. It has been postulated that tunnels found in trHbs serve as an alternative diffusion path for ligands. In case of *Syn*Hb, there is no tunnel observed and subsequently, three possibilities are proposed for ligand entry and exit which includes: 1) Ligands enter and exit directly from the heme pocket through the solvent face. 2) Ligands enter through the solvent face of the heme pocket, and then a tunnel formation in the ligand bound state facilitate the ligand exit. 3) Ligand entry and exit pathways are not clear in *Syn*Hb structures.

3. Proposed physiological function of *Synechocystis* hemoglobin

The new class of Hb differs from the classical Hbs in their cellular location, primary sequence, expression pattern, three-dimensional fold, heme pocket architecture, ligand binding characteristics, heme pocket electrostatics etc. and thus, forced the researchers worldwide to re-investigate their functions which might diverge widely during evolution of globins [11, 44]. Based on several reports, the oxygen transport and storage function which are usually associated with hemoglobins have been ruled out and various other functions including detection, scavenging, and detoxification of O_2 and O_2- derived species (e.g. NO and CO) have been proposed.

Despite numerous efforts, physiological function for *Syn*Hb is still not clear. Several functions have been proposed that vary from reversible binding of diatomic ligands to redox reactions, to peroxidase activity, and nitrite and hydroxylamine chemistry [23, 24, 45–48]. The possibility for *Syn*Hb to function as an oxygen transporter protein is excluded as the oxygen dissociation rate constant of *Syn*Hb is too low for its involvement in the facilitated diffusion of oxygen, thus suggesting that it may be involved in

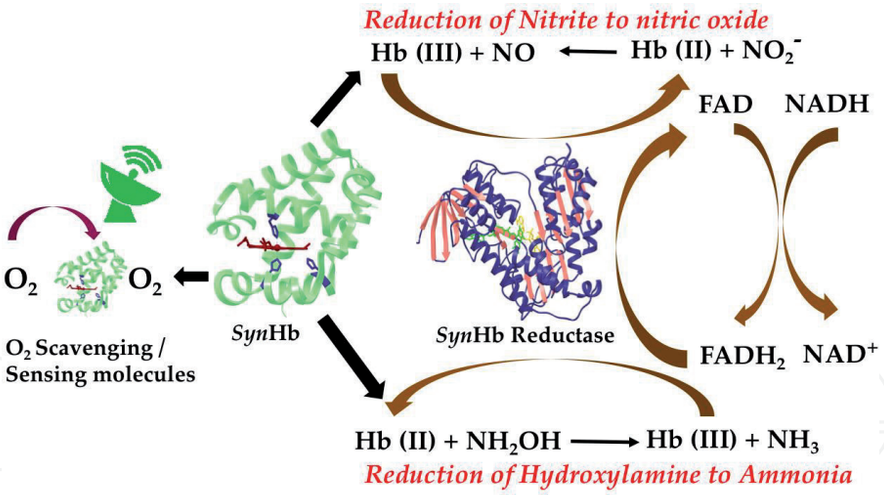

Figure 5.
Proposed working model of Synechocystis hemoglobin-reductase complex.

O_2 scavenging or sensing mechanism [25, 37]. In cyanobacteria, the metabolism of N_2 is different and assimilates nitrogen through the reduction of nitrate under conditions ranging from normoxic to hypoxic [24]. It has been shown that at low oxygen concentration, cyanobacteria can accumulate nitrate and nitrite to a very high concentration in the range of millimolar [49]. Sturms et al., demonstrated that the ferrous *Syn*Hb showed nitrite reduction rate 10 times faster than the animal hemoglobins [24], which indicates that cyanobacterial Hb could serve as an anaerobic nitrite reductase *in vivo*. Similarly, the same group has shown that the hydroxylamine reduction rate to ammonia is 100–2500 times faster than animal hemoglobins *in vitro* [23], which supports the hypothesis that it contribute to anaerobic nitrogen metabolism in support of anaerobic respiration and survival during hypoxia. Another existing study suggested that *Syn*Hb can serve as nitric oxide dioxygenase (NOD) enzyme by substituting for flavohemoglobins (FHbs) in *E. coli* during NO challenge [26]. In an artificial reduction system, oxyferrous-*Syn*Hb can react rapidly with NO and subsequently scavenge NO with a controlled reduction rate *in vitro*. Interestingly, it has been in *Synechococcous* sp. PCC 7002, the close relative of *Synechocystis* sp. PCC 6803, that globin null mutant strain experienced more stress under NO exposure compared to wild type strain [50]. These NO protection/scavenging functions would thus need the requirement of a suitable reduction mechanism that converts inactive ferric state of *Syn*Hb back to active ferrous state of *Syn*Hb to accomplish these functions *in vivo*. These studies prompted other researchers to search for related reductase protein for *Syn*Hb which can perform the proposed nitrite reductase function. Recently, Uppal et al., reported a protein in *Synechocystis* sp. PCC 6803 which was annotated as dihydrolipoamide dehydrogenase (DLDH) in the database. Their studies clearly showed that the putative cognate reductase "named as *Synechocystis* Hb reductase (*Syn*HbRed)" interact and reduce the Fe^{3+}-*Syn*Hb back to active Fe^{2+}-*Syn*Hb *in vitro* [51]. Based on these results, we proposed a hypothetical *Synechocystis* hemoglobin-reductase reduction system as shown in **Figure 5**.

4. Biotechnological applications of *Synechocystis* hemoglobin

4.1 In designing a stable hemoglobin-based blood substitute using protein engineering approach

In the last few decades, there is a significant progress in the development of oxygen-carrying blood substitutes. Since the 1980s, human blood substitutes have

been in the pipeline in the medical and life science research fields [52]. Currently, there are none in the market because of scientific and political reasons. There are a few blood substitutes still progressing through clinical trials, and the academic community is still actively improving the products, also known as oxygen therapeutics and hemoglobin-based oxygen carriers [53, 54]. Over the last few years, studies have focused on developing "recombinant hemoglobin-based oxygen carriers" (rHBOCs) which can be used as an alternative to blood during transfusion therapy. Recombinant human hemoglobin is produced in heterologous expression systems like *E. coli* to fulfill the need for artificial blood substitutes [55–59]. However, such hemoglobins suffer quite a few disadvantages like dissociation into dimers, poor stability, easy clearance from circulation, high blood pressure, poor expression yields, improper ligand affinities and fast heme dissociation [60]. Recently, protein engineering approaches have been employed for designing more stable Hb-based blood substitutes, with several properties improved. Several laboratories are intensively involved to tackle the major remaining problems associated with artificial blood substitutes like stability and rapid heme dissociation [61]. Mb has been invariably used as a model protein for the commercial development of blood substitutes [62]. This small, stable, and well-studied Hb thus became a gold standard for protein engineering approaches.

The newly discovered truncated and hexacoordinate globins exhibit unique features that allow the exploration of a whole range of proteins, some of which might be more stable than Mb, thus allowing newer ways for comparative mutagenesis strategies to improve stability. *Synechocystis* hemoglobin with its unique His-heme linkage and unparalleled stability serves as an excellent reference system. Thus, the major application of *Synechocystis* hemoglobin is in the designing of stable hemoglobin-based blood substitutes, an area of translational science in hemoglobin biotechnology. It has been assumed that introduction of extra covalent linkage via histidine to heme in other globin might allow a new strategy to enhance heme stability. Lecomte's group was successful in engineering covalent linkage itself by substituting Leu79 to His and demonstrated that single variant L79H/H117A bound the heme weakly but nonetheless formed a covalent linkage between His79Nε2-heme 2-vinyl atom, analogous to His117-heme 2-vinyl linkage [27]. Another successful attempt to engineer the heme stability was done by Uppal et al., in myoglobin as a first step toward the production of a stable hemoglobin-based oxygen carriers [22]. Their work clearly demonstrated that the Mb mutant (I107H) with the engineered covalent linkage holds heme tightly, stable to denaturants and exhibited ligand binding kinetics similar to wild-type protein. The future perspective of this work is to engineer the extra covalent linkage in recombinant human Hb, necessitating a step toward the production of stable hemoglobin-based oxygen carriers.

4.2 *Synechocystis* hemoglobin as a fusion tag for enhancing the expression, solubility and purification of other proteins

Recent years have witnessed tremendous increase in the number of tags and the development of fusion strategies to facilitate the expression, purification, and solubilization of recombinant proteins which can be used as industrial enzymes, for drug discovery, and biotherapeutics [63]. There are now a wide variety of fusion tags available in the market which are well-characterized and used in the biotechnological industry to obtain highly purified biologically active recombinant proteins [64]. However, these available tags have a major limitation, i.e., the absence of any color to facilitate the visualization of target protein during the expression and purification process. Hemoglobins because of their distinctive bright red color, high solubility and stability offer a unique advantage of tracking of the target

fused protein during expression and at different steps of purification and even in crystallization and thus minimizing the cost and time in fusion protein technology. Previous reports showed the use of visible tag systems such as flavoenzymes and hemeproteins that contain colored chromophores [65]. The *Vitreoscilla* hemoglobin (VHb) from the bacterium *Vitreoscilla* has been used successfully as a fusion expression vector for the production of many target proteins [66]. Based on recent biochemical studies of newly discovered hemoglobins, it is revealed that *Synechocystis* hemoglobin is the well-suited protein for high-throughput protein expression and purification [67]. Since it is small, very stable, highly soluble with a high expression yield, it can improve the expression yield and solubility of the desired protein.

5. Conclusion

In the last few decades, an intense research effort from several researchers worldwide has enabled us to uncover the unique properties of *Synechocystis* hemoglobin and shed the light on its physiological function. Despite extensive information available for *Syn*Hb, there are still many unanswered questions that need to be investigated. Moreover, the evolutionary significance of the third His-heme covalent linkage and its role in the physiological function of *Syn*Hb is still unclear. However, *Synechocystis* Hb presents an important model system to understand protein folding and stability in general. Furthermore, the knowledge gained from mutational and expression studies in *Synechocystis* Hb can be applied to other globins e.g. human hemoglobin for designing an efficient oxygen delivery vehicle.

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Conflict of interest

The authors declare no conflict of interest.

Abbreviations

GlbN	Cyanoglobin
HxHbs	Hexacoordinate hemoglobin
FHb	Flavohemoglobin
Hb	Hemoglobin
*Syn*Hb	*Synechocystis* hemoglobin
NMR	Nuclear magnetic resonance
NOD	Nitric oxide dioxygenase
nsHb	Non-symbiotic hemoglobin

PDB	Protein data bank
TrHb	Truncated hemoglobin
O_2	Oxygen
CO	Carbon monoxide
NO	Nitric oxide
HBOCs	Hemoglobin based oxygen carriers

Author details

Mohd. Asim Khan[1], Sheetal Uppal[2] and Suman Kundu[1*]

1 Department of Biochemistry, University of Delhi South Campus, New Delhi, India

2 National Eye Institute, National Institutes of Health, Bethesda, MD, USA

*Address all correspondence to: suman.kundu@south.du.ac.in

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